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短　報
Note

Characterization of Transgenic Tobacco Plants Expressing the *hvnas1* Open Reading Frame under the Control of the *AtXyn1* Promoter

By

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Summary : In order to express nicotianamine (NA) synthase in tobacco plants, particularly in their vascular bundles, we introduced the *AtXyn1* promoter : : Ω-*hvnas1* open reading frame cassette into tobacco plants. The accumulation of NA was increased, particularly in the young leaves of transgenic tobacco plants. The Ni tolerance of these plants was slightly higher than that of vector control plants. The Fe content of the whole shoots of the *AtXyn1* promoter : : Ω-*hvnas1* transgenic tobacco plants increased under normal conditions regardless of NA contents, compared with the vector control plants.

Key words : *AtXyn1, hvnas1,* nickel tolerance, nicotianamine, transgenic tobacco

1. Introduction

Although the functions of trace essential metals and the high levels of stress they cause in plants have long been subjects of investigation[1], the localization and homeostasis of these metals in the tissues of higher plants are only recently being studied. More specifically, considerable attention has been focused on the physiological functions of nicotianamine (NA)—a chelator of divalent transition metals that is distributed among higher plants[2–4]. NA has been suggested to mediate the translocation of Fe in the phloem and Cu and Zn in the phloem and xylem[5]. Transporters of the yellow stripe-like (YSL) family, which transport NA-metal complexes, have been identified in higher plants[6–9]. The detoxification of Ni by NA has also been reported[10–12]. Moreover, NA plays an important role in the hyperaccumulation of Ni[13,14].

Young plant tissues in which rapid cell division and differentiation occur generally require a continuous and adequate supply of essential transition metals, which are delivered via the phloem. Thus, the localization and transport of NA is crucial for the homeostasis of transition metals and the development in higher plants. In this study, we expressed the open reading frame (ORF) of the barley nicotianamine synthase (NAS) gene *hvnas1*[15] under the control of the *AtXyn1* promoter in tobacco. *AtXyn1,* which has been isolated from *Arabidopsis thaliana,* encodes a xylanase and is predominantly expressed in vascular bundles of the young leaf[16]. The NA content, particularly in the young leaves, was higher in transgenic tobacco plants expressing *hvnas1* than in tobacco plants expressing *uidA*. We will discuss the increased Fe content of the shoots accompanied by local and rather small increase of NA content.

2. Materials and Methods

2.1 Construction of binary vectors and transformation

A cassette containing the ORF of *hvnas1*[15] and the 3′ untranslated region (UTR) of the nopaline synthase

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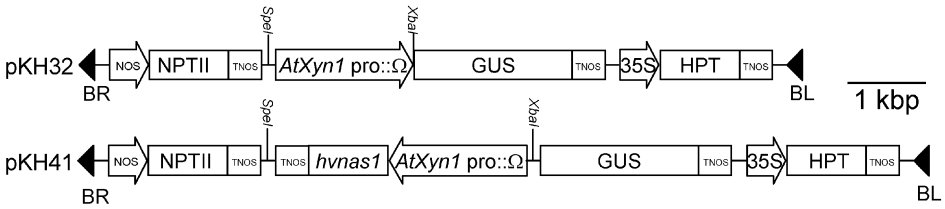

Fig. 1 The constracts of the *hvnas1* ORF under the control of the *AtXyn1* promoter. T-DNA regions of pKH32 and pKH41. Abbreviations : BR, right border ; BL, left border ; NPTII, neomycin phosphotransferase ; GUS, b-glucuronidase ; HPT, hygromycin phosphotransferase ; NOS, nopaline synthase promoter ; 35S, *CaMV35S* promoter ; TNOS, 3′ UTR of the nopaline synthase gene.

gene was cloned into pBluescript SKII (+) between the NcoI and EcoRI sites. The plasmid DNA sequence containing the promoter region of *AtXyn1*[16] was amplified by using polymerase chain reaction (PCR). Two primers were used : Xyn1pro5, 5′-GAGAGAGAG-CGGCCGCTAATATACTAGCCATTTGAATAAAG-3′ and Xyn1pro3omega, 5′-GAGATCTAGATGTAATTG-TAAATAGTAATTGTAATGTTGTTTGTTGTTTGT-TGTTGTTGGTAATTGTTGTAAAAATATTCTT-ATGTATTTTGTTTGAG-3′. The underlined sequence indicates the Ω element which enhances the expression of foreign gene transcripts[17] (accession no. M 31471). An *AtXyn1* promoter : : Ω cassette was subcloned into the region upstream of the ORF of the *uidA* gene, between the SpeI (originally HindIII) and XbaI sites of pIG121Hm[18] ; this vector was designated as pKH32 (Fig. 1). An *AtXyn1* promoter : : Ω cassette was subcloned into the region upstream of the ORF of *hvnas1*. A cassette containing the *AtXyn1* promoter : : Ω, the ORF of the *hvnas1* gene, and the 3′ UTR of the nopaline synthase gene was cloned between the XbaI and SpeI (originally HindIII) sites of pIG121Hm ; this vector was designated as pKH41 (Fig. 1). Transformation of the vectors into tobacco plants and regeneration were performed by using the standard leaf-disc transformation method[19].

2.2 Plant material, growth conditions, and treatment with heavy metals

Seeds of T2 homozygous transgenic plants were surface sterilized and sown on Murashige and Skoog (MS) basal medium[20]. The tobacco seedlings were grown under a 16-h/8-h light/dark cycle for 3 weeks at 25℃. The seedlings were then cultivated hydroponically in a green house at 27℃ with natural light. The seedlings were grown for 4 d in a culture solution containing 10 μM Ni ($NiSO_4$), and then for 10 d in a solution containing 50 μM Ni. The composition of the culture solution has been described by MARUYAMA *et al.*[21].

2.3 Determination of metal contents

Shoots were harvested and dried for 3–4 d at 60℃, and the dried leaves were digested for 12 h in HNO_3 solution at 120℃ and dissolved in 10 mL 1 N HCl. The metal contents were determined with an atomic absorption spectrometer (AA-680 or AA-670 ; Shimadzu Co. Ltd., Japan).

2.4 Extraction and measurement of endogenous NA

Hydroponically cultured tobacco plants (with 6–9 leaves) were harvested. The first and second young leaves of the plants (young leaves), and the largest and second largest leaves (mature leaves) were separated and stored at −80℃. The frozen plant materials were homogenized in liquid nitrogen with a mortar and pestle ; the materials were then thawed by mixing with 20 volumes of deionized water (w/v). The samples were heated to 80℃ for 20 min and centrifuged. The supernatants were analyzed by high performance liquid chromatography (HPLC). HPLC was performed under the following conditions, described by Le JEAN *et al.*[22]. An octadecyl silane (ODS) column (TSK-gel ODS-100V ; 5μm ; Φ, 4.6 mm×250 mm ; Tosoh, Japan) was used. The HPLC system composed of the DGU-20A3, LC-20AB, CTO-6A, and RF-10A_{XL} units (Shimadzu Co. Ltd.).

3. Results and Discussion

3.1 NA contents of transgenic tobacco plants

NA is considered to play a crucial role in the transport of transition metals via vascular bundles ; therefore, we aimed to characterize transgenic plants that expressed NAS, particularly in the vascular bundles of young leaf. The *AtXyn1* promoter region[16] was used for this purpose. We introduced the *AtXyn1* promoter : : Ω-*uidA* (pKH32) and *AtXyn1* promoter : : Ω-*hvnas1* ORF (pKH 41) cassettes into tobacco plants (Fig. 1).

We measured the amount of NA accumulated in the transgenic tobacco plants (Fig. 2) and also in tobacco plants expressing the CaMV35S promoter : : *NAS*

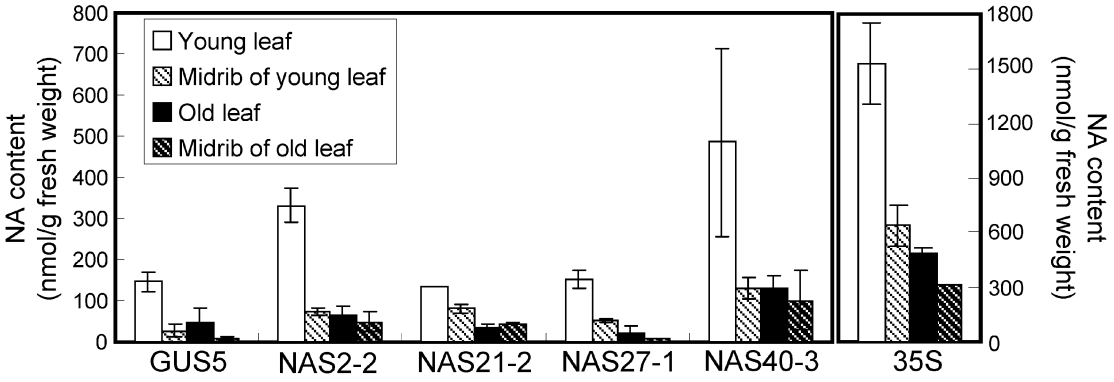

Fig. 2 NA content of transgenic tobacco plants.
Extracts were prepared from the midribs and other parts of the leaves without midribs (denoted by young leaf or old leaf) of hydroponically cultured tobacco plants (with 6–9 leaves), and the extracts were analyzed by HPLC. GUS5 denotes transgenic tobacco plants expressing pKH32. NAS2-2, NAS21-2, NAS27-1, and NAS40-3 denote transgenic tobacco plants expressing pKH41. Transgenic tobacco plants expressing the *CaMV35S* promoter : : *hvnas1* (KIM *et al.* 2005) cassette are denoted by 35S. Each bar represents the mean± standard error (n =3).

cassette[11]. The NA content of the young leaves was higher than that of the old leaves in the case of the 35 S : : *NAS* and GUS lines. These findings are consistent with previous reports on non-transgenic plants[23,24]. The NA content was increased in the NAS2-2 and NAS 40-3 lines of transgenic pKH41-expressing tobacco plants, particularly in young leaves. The NA content was higher in the leaves without midribs than in midribs. However, the ratio of the NA content in the midribs to that in the leaves without midribs was increased in pKH41- and CaMV35S promoter : : *NAS*-expressing tobacco plants. In case of NAS21–2 and NAS27–1 lines, NA contents in the young leaf without midrib were the same as that in GUS5 line, whereas those in the midrib of young leaf were higher than that in GUS5 line. The NA content was lower in the pKH41 -expressing tobacco plants than in the 35S : : *NAS*-expressing tobacco plants.

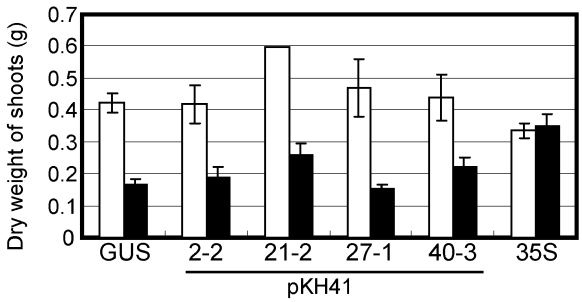

Fig. 3 The growth of transgenic tobacco plants.
Dry weights of the shoots of transgenic tobacco plants (with 6–9 leaves) grown under hydroponic conditions in the presence or absence of Ni (see Materials and Methods). The transgenic lines indicated in Fig. 2 were used. Open bar : without Ni, black bar : with Ni. Each bar represents the mean±standard error (n=3).

3.2 Metal contents of transgenic tobacco plants

We examined Ni tolerance of hydroponically grown transgenic tobacco (Fig. 3). The decrease in the dry weights of shoots in response to Ni stress was compared among the transgenic tobacco lines. The dry weights of the shoots of Ni-treated plants expressed as a percentage of the dry weights of the shoots of the control plants were as follows : GUS line, 40% ; NAS2–2 line, 45% ; NAS40–3 line, 52% ; and 35S line, 100%. These percentages were highly correlated with the NA content of the young leaves without midribs (R^2=0.98), thus confirming that the accumulation of NA contributes to the detoxification of Ni.

The Ni content of the shoots of transgenic tobacco plants grown in media supplemented with Ni was almost identical among transgenic lines (Fig. 4a). Thus, the increase in NA by itself was not enough to cause an increase in the Ni content. As observed in *Thlaspi caerulescens*[13,14], enhanced circulation of NA may also be required for the accumulation of large amounts of Ni in tobacco.

The Fe content was higher in the shoots of the pKH41- and 35S-expressing tobacco plants grown in the absence of Ni than in GUS-expressing tobacco plants (Fig. 4b). This finding is consistent with that of a previous report[11]. However, the correlation between the NA and

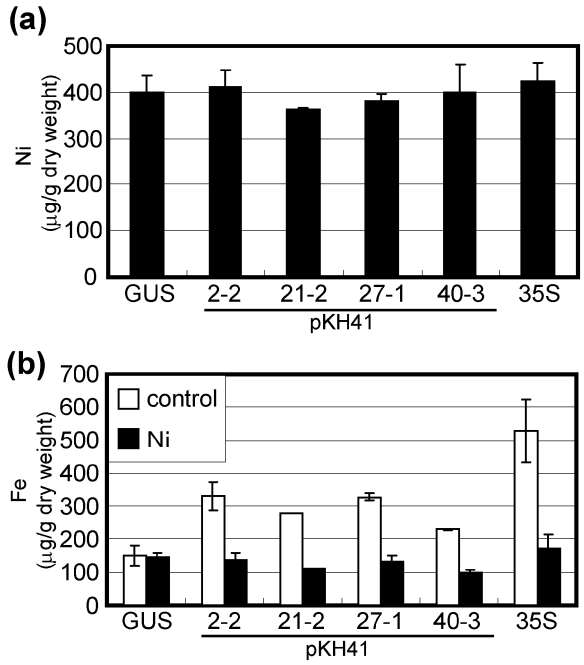

Fig. 4 Ni and Fe contents of the shoots of transgenic tobacco plants.
(a) Ni content of the shoots of transgenic tobacco plants. The plants indicated in Fig. 3 that were grown in the presence of Ni were analyzed. Each bar represents the mean±standard error ($n=3$). (b) Fe content of the shoots of transgenic tobacco plants. The plants indicated in Fig. 3 were analyzed. Open bar : without Ni, black bar : with Ni. Each bar represents the mean±standard error ($n=3$).

Fe contents was low. Considering the distribution of NA, the NA contents in the midrib of the young leaves from all of pKH41-expressing tobacco plants were higher than that from GUS5 plants (Fig. 2, light hatched bars). Thise increase of NA contents may contribute to the increase of Fe contents. The distribution pattern of Fe did not vary with the position of the leaves (data not shown). It is well known that Fe recirculation is inefficient in plant body, and the old leaves often accumulate Fe[1]. Thus small increase of NA in the vascular bundles of the young leaf may enhance Fe uptake by mesophyll cells in the young leaf through the growth period, then Fe content in the whole shoot may be increased due to local small increase of NA.

The Fe contents in the shoots of pKH41- and 35 S-expressing tobacco plants grown in the presence of Ni were almost identical to those in the shoots of GUS-expressing tobacco plants (Fig. 4b black bars). At high concentrations, NA may trap Ni ions which in turn may prevent an increase in the Fe content.

4. Conclusion

In the pKH41-expressing tobacco lines, the NA contents of the young leaves especially in the midribs and the Fe contents of the whole shoot were increased. It is thought that Fe is primarily transported along with citrate via the xylem and with NA via the phloem[5]. Generally, developing young leaves function as sink organs, and nutrients are supplied to these leaves primarily via the phloem. The increase in the NA contents of young leaves and the relative NA content of the midribs may enhance the supply of Fe to cells of the young leaves. It is cost-effective that rather small increase of NA is enough to increase Fe in plant body, because biosynthesis of one molecule of NA consumes 3 molecules of ATP and 3 molecules of assimilated N[15].

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オオムギのニコチアナミン合成酵素遺伝子を維管束特異的プロモーターと共に導入した形質転換タバコの解析

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要約：ニコチアナミン合成酵素をタバコの維管束で特に発現させるために，シロイヌナズナのキシラナーゼ遺伝子（*AtXyn1*）のプロモーター―オメガ配列―オオムギのニコチアナミン合成酵素遺伝子（*hvnas1*）の翻訳領域をつないだものをタバコに導入した。ニコチアナミンの蓄積量は形質転換タバコの特に若い葉で増加した。これら形質転換体のニッケル耐性はベクターのみで形質転換したものよりわずかに高かった。ニコチアナミン含量にかかわらず，*hvnas1* を導入したタバコの地上部全体の鉄含量は通常の栽培条件でベクターのみのものに比べて増加した。

キーワード：*AtXyn1, hvnas1,* ニッケル耐性，ニコチアナミン，形質転換タバコ

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